

**Application
for
United States Letters Patent**

To all whom it may concern:

Be it known that Paul B. Fisher

have invented certain new and useful improvements in

RECIPROCAL SUBTRACTION DIFFERENTIAL DISPLAY

of which the following is a full, clear and exact description.

RECIPROCAL SUBTRACTION DIFFERENTIAL DISPLAY

5 This application is a continuation-in-part of U.S. Serial
No. 09/197,889, filed November 23, 1998, which is a
continuation-in-part of U.S. Serial Application No.
09/185,115, filed November 3, 1998 which is a
continuation-in-part of U.S. Serial Application No.
10 09/032,684, filed February 27, 1998. The content of the
above identified applications are hereby incorporated
into this application by reference.

15 Throughout this application, various references are
referred to within parentheses. Disclosures of these
publications in their entireties are hereby incorporated
by reference into this application to more fully describe
the state of the art to which this invention pertains.

20 Background of the Invention

Changes in gene expression are important determinants of
normal cellular physiology, including cell cycle
regulation, differentiation and development, and they
directly contribute to abnormal cellular physiology,
25 including developmental anomalies, aberrant programs of
differentiation and cancer (1-4). In these contexts,
the identification, cloning and characterization of
differentially expressed genes will provide relevant and
important insights into the molecular determinants of
30 processes such as growth, development, aging,
differentiation and cancer. A number of procedures can
be used to identify and clone differentially expressed
genes. These include, subtractive hybridization (5-10),
differential RNA display (DDRT-PCR) (3,4, 11,12), RNA
35 fingerprinting by arbitrarily primed PCR (RAP-PCR)
(13,14), representational difference analysis (RDA) (15),
serial analysis of gene expression (SAGE) (16,17),
electronic subtraction (18,19) and combinatorial gene
matrix analyses (20).

Since first introduced by Liang and Pardee (11), DDRT-PCR has gained wide popularity in analyzing and cloning differentially expressed genes. In DDRT-PCR, total RNAs or mRNAs from two or more cell types (or cells grown under different conditions, cells representing different stages of development, cells treated with agents modifying cellular physiology, etc.) are reverse-transcribed with two-base-pair anchored oligo dT primers, which divide mRNA populations into 12 cDNA subgroups. Then, each cDNA subgroup is amplified by PCR with one of 20 arbitrary 10-mer 5' primers and a 3' anchored primer and the PCR-amplified cDNA fragments are resolved in DNA sequencing gels. The combinations of primers are designed not only to yield a detectable size and number of bands, but also to display nearly the complete repertoire of mRNA species.

DDRT-PCR is a powerful methodology in which a vast number of mRNA species (>20,000, if no redundancy occurs) can be analyzed with only a small quantity of RNA (about 5 μ g) (11). DDRT-PCR is often the method of choice when the RNA source is limiting, such as tissue biopsies. A direct advantage of DDRT-PCR is the ability to identify and isolate both up- and down-regulated differentially expressed genes in the same reaction. Furthermore, the DDRT-PCR technique permits the display of multiple samples in the same gel, which is useful in defining specific diagnostic alterations in RNA species and for temporally analyzing gene expression changes. However, the DDRT-PCR technique is not problem free. Difficulties encountered when using standard DDRT-PCR include, a high incidence of false positives and redundant gene identification, poor reproducibility, biased gene display and lack of functional information about the cloned cDNA. Furthermore, poor separation can mask differentially expressed genes of low abundance under the intense signals generated by highly expressed genes. The

generation of false positives and redundancy can be highly problematic, resulting in an inordinate expenditure of resources to confirm appropriate differential expression and uniqueness of the isolated
5 cDNAs. The cDNAs must be isolated from the gels in pure form (contamination of bands with multiple sequences complicates clone identification), reamplified, placed in an appropriate cloning vector, analyzed for authentic differential expression and finally sequenced. These
10 limitations of the standard DDRT-PCR approaches emphasize the need for improvements in this procedure to more efficiently and selectively identify differentially expressed genes.

15 A number of modifications and improvements of the DDRT-PCR approach have been described (21-23). Single anchor or degenerate two-base anchor oligo dT primers can be used to streamline the massive numbers of reverse transcription and PCR reactions required for validation
20 of cDNAs as well as to reduce false positives (24,25). Reproducibility can be improved by lengthening the arbitrary 5' primers to accommodate a convenient restriction site followed by two cycles of PCR with successive low- and high-stringency annealing
25 temperatures (25,26). DDRT-PCR with inosine-containing 5' arbitrary primers can also increase reproducibility of this approach (27). However, since these modifications have only been analyzed using a subset of primers, further studies are necessary to validate these
30 modifications of DDRT-PCR with additional primers and in several model systems.

In addition to genomic DNA contamination, mispriming, PCR artifacts, the high incidence of false positives and
35 redundancy is also ascribed to poor separation between bands and the complexity of the templates amplified (28). Furthermore, poor separation can mask differentially

expressed genes of low abundance under the intense signals generated by highly expressed genes. By enriching for unique cDNAs and removing common ones, it should in principle be possible to enrich for low abundant gene products and significantly decrease the complexity of amplified sequences. In addition, the sequence bias of DDRT-PCR should also be reduced by decreasing template complexity. These assumptions serve as the basis for the development of reciprocal subtraction differential RNA display (RSDD).

Subtractive hybridization, in which hybridization between tester and driver is followed by selective removal of common gene products, enriches for unique gene products in the tester cDNA population and reduces the abundance of common cDNAs (9). A subtracted cDNA library can be analyzed to identify and clone differentially expressed genes by randomly picking colonies or by differential screening (29-31). Although subtractive hybridization has been successfully used to clone a number of differentially expressed genes (5-7,10), this approach is both labor-intensive and does not result in isolation of the full spectrum of genes displaying altered expression (9,18).

In principle, DDRT-PCR performed with subtracted RNA or cDNA samples represents a powerful strategy to clone up and down-regulated gene products. This approach should result in the enrichment of unique sequences and a reduction or elimination of common sequences. This scheme should also result in a consistent reduction in band complexity on a display gel, thereby permitting a clearer separation of cDNAs resulting in fewer false positive reactions. Additionally, it should be possible to use fewer primer sets for reverse transcription and PCR reactions to analyze the complete spectrum of differentially expressed genes. Of particular importance

for gene identification and isolation, rare gene products that are masked by strong common gene products should be displayed by using subtraction hybridization in combination with DDRT-PCR. In addition, the DDRT-PCR approach with subtractive libraries could also prove valuable for efficiently screening subtracted cDNA libraries for differentially expressed genes. However, even though subtraction hybridization plus DDRT-PCR appears attractive for the reasons indicated above, a previous attempt to use this approach has proven of only marginal success in consistently reducing the complexity of the signals generated, compared with the standard DDRT-PCR scheme (32).

We presently describe a reciprocal subtraction differential RNA display (RSDD) approach that efficiently and consistently reduces the complexity of DDRT-PCR and results in the identification and cloning of genes displaying anticipated differential expression.

20

Summary of the Invention

This invention provides a method for identifying differentially expressed nucleic acids between two samples, comprising: (a) selecting a first and second
5 nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) performing reciprocal subtraction between the nucleic acid samples to produce two subtracted nucleic acid samples; (c) amplifying the two subtracted nucleic acid samples; and
10 (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

This invention also provides a method for identifying differentially expressed nucleic acids between two
15 samples, comprising: (a) selecting a first and second nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) amplifying the two nucleic acid samples; (c) performing reciprocal subtraction between the amplified nucleic acid samples to
20 produce two subtracted nucleic acid samples; and (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

This invention further provides the above-described
25 methods, wherein the first and second nucleic acid samples are obtained from cells in different developmental stages.

This invention further provides the above-described
30 methods, wherein the first and second nucleic acid samples are obtained from cells from different tissue types.

Also, this invention provides the above-described
35 methods, wherein the 3' primer used in the PCR amplification is an oligo dT 3' primer.

In addition, this invention provides the above-described methods, wherein the 3' primer used in the PCR amplification is a single anchor oligo dT 3' primer.

- 5 This invention also provides the above-described methods, wherein the comparing of step (e) comprises using a gel to separate the nucleic acids from both of the libraries.

- 10 This invention provides the isolated nucleic acid identified by the the above-described methods, wherein the nucleic acid was not previously known to be differentially expressed between the two samples.

Brief Description of the Figures

Figure 1

Identification of differentially expressed sequence tags using reciprocal subtraction differential RNA display (RSDD). Left panel: differential RNA display pattern of conventional DDRT-PCR with RNA from E11 (C) and E11-NMT (T) cells and an RSDD analysis of reciprocally subtracted E11 minus E11-NMT (C/T) and E11-NMT minus E11 (T/C) cDNA libraries. Right panel: representative RSDD patterns using different sets of primers.

Figure 2

Reverse Northern analysis of differentially expressed sequence tags identified by reciprocal subtraction differential RNA display (RSDD). Differentially expressed sequence tags obtained from RSDD were dot-blotted onto Nylon membranes and probed with ^{32}P -cDNA reverse transcribed from RNA samples of E11 and E11-NMT cells.

Figure 3A

Differential expression of representative progression elevated genes (PEGen) and progression suppressed genes (PSGen) identified by reciprocal subtraction differential RNA display (RSDD) and reverse Northern blotting. Northern blots of E11 and E11-NMT RNA samples were probed with radiolabeled (^{32}P) expressed sequence tags identified by RSDD and reverse Northern blotting.

Figure 3B

Differential expression of representative progression elevated genes (PEGen) and progression suppressed genes (PSGen) identified by reciprocal subtraction differential RNA display (RSDD) and reverse Northern blotting.

Figure 4

Differential expression of representative progression

elevated genes (PEGen) and progression suppressed genes (PSGen) identified by reciprocal subtraction differential RNA display (RSDD) and reverse Northern blotting. Northern blots of cells displaying various stages of transformation progression were probed with radiolabeled (³²P) expressed sequence tags identified by RSDD and reverse Northern blotting. The cell types used include, Unprogressed E11 (-), CREFE11-NMT F1 (-) and CREFE11-NMT F2 (-) somatic cell hybrids, E11xE11-NMT A6 (-) somatic cell hybrid, E11xE11-NMT 3b (-) somatic cell hybrid, and E11-NMT Aza B1 (-) and E11-NMT Aza C1 (-) 5-azacytidine treated E11-NMT clones; and Progressed E11-NMT (+), CREFE11-NMT R1 (+) and CREFE11-NMT R2 (+) somatic cell hybrids, E11xE11-NMT A6TD (+) nude mouse tumor derived somatic cell hybrid, E11xE11-NMT IIa (+), E11-Ras R12 (+) a Ha-ras transformed E11 clone and E11-HPV E6/E7 (+) an E11 clone transformed by the E6 and E7 region of HPV-18.

20 Figure 5

cdNA fragment of PEGen 7 - 90% Homology to Human HPV16 E1BP. (Sequence ID No. 1)

Figure 6

25 cdNA fragment of PEGen 8 - Rat phosphofructose kinase C. (Sequence ID No. 2)

Figure 7

30 First (Sequence ID No. 3) and second (Sequence ID No. 4) cdNA fragments of PEGen 13.

Figure 8

cdNA fragment of PEGen 14. (Sequence ID No. 5)

35 Figure 9

cdNA fragment of PEGen 15. (Sequence ID No. 6)

Figure 10

cDNA fragment of PEGen 21 which has 94% homology to mouse
FIN 14. (Sequence ID No. 7)

5 Figure 11

cDNA fragment of PEGen 24. (Sequence ID No. 8)

Figure 12

10 cDNA fragment of PEGen 26 - Rat poly ADP-ribose
polymerase. (Sequence ID No. 9)

Figure 13

cDNA fragment of PEGen 28. (Sequence ID No. 10)

15 Figure 14

cDNA fragment of PEGen 42. (Sequence ID No. 11)

Figure 15

20 cDNA fragment of PEGen 43. (Sequence ID No. 12)

Figure 16

cDNA fragment of PEGen 44. (Sequence ID No. 13)

Figure 17

25 cDNA fragment of PEGen 48. (Sequence ID No. 14)

Figure 18

30 cDNA fragment of PSGen 1 which has 80% homology to *B.*
taurus supervillin. (Sequence ID No. 15)

Figure 19

cDNA fragment of PSGen 2 which has 91% homology to human
HTLV-1 Tax interacting protein. (Sequence ID No. 16)

35 Figure 20

cDNA fragment of PSGen 4 - Rat proteasome activator.
(Sequence ID No. 17)

Figure 21

cDNA fragment of PSGen 10 - Rat Ferritin Heavy Chain.
(Sequence ID No. 18)

5 Figure 22

cDNA fragment of PSGen 12. (Sequence ID No. 19)

Figure 23

cDNA fragment of PSGen 13. (Sequence ID No. 20)

10

Figure 24

cDNA fragment of PSGen 23. (Sequence ID No. 21)

Figure 25

15 cDNA fragment of PSGen 24. (Sequence ID No. 22)

Figure 26

cDNA fragment of PSGen 25. (Sequence ID No. 23)

20 Figure 27

cDNA fragment of PSGen 26.

Figure 28

cDNA fragment of PSGen 27.

25

Figure 29

cDNA fragment of PSGen 28.

Figure 30

30 cDNA fragment of PSGen 29.

Figure 31

cDNA fragment of PEGen 32.

35

Figure 32

Schematic outline of the reciprocal differential RNA display (RSDD) protocol. This scheme incorporates three steps, reciprocal subtraction of cDNA libraries, differential display of *in vivo* excised cDNAs and expression analysis by reverse Northern and standard Northern blotting. For the present application of RSDD, reciprocal subtraction hybridization was performed using libraries constructed from E11 and E11-NMT cells, i.e., E11 minus E11-NMT and E11-NMT minus E11. Differentially expressed cDNAs identified on gels using differential RNA were isolated, reamplified and analyzed for expression by reverse Northern blotting. To confirm differential expression cDNAs were analyzed using Northern blotting.

Figure 33

Differential expression of representative progression elevated (PEGen) and progression suppressed genes (PSGen) identified by RSDD and reverse Northern blotting. Northern blots of E11 and E11-NMT RNA samples were probed with radiolabeled (^{32}P) expressed sequence tags identified by RSDD and reverse Northern blotting. Equal loading of E11 and E11-NMT RNA is demonstrated by ethidium bromide (EtBr) staining.

Figure 34

Differential expression of representative PEGen and PSGen genes identified by RSDD and reverse Northern blotting in a large panel of rodent cells displaying differences in transformation progression. Northern blots of cells displaying various stages of transformation progression were probed with radiolabeled (^{32}P) expressed sequence tags identified by RSDD and reverse Northern blotting. The cell types used include: Unprogressed E11 (-), CREF X E11-NMT F1 (-) and CREF X E11-NMT F2 (-) somatic cell hybrids, E11 X E11-NMT A6 (-) somatic cell hybrid, E11 X E11-NMT 3b (-) somatic cell hybrid, and E11-NMT AZA B1

(-) and E11-NMT AZA C1 (-) 5-azacytidine-treated E11-NMT clones; and Progressed E11-NMT (+), CREF X E11-NMT R1 (+) and CREF X E11-NMT R2 (+) somatic cell hybrids, E11 X E11-NMT A6TD (+) nude mouse tumor derived somatic cell
5 hybrid, E11 X E11-NMT IIa (+), E11-Ras R12 (+) and E11-HPV E6/E7 (+) an E11 clone transformed by the E6 and E7 region of HPV-18. Equal loading of RNAs is demonstrated by ethidium bromide (EtBr) staining.

10 Figure 35 A

PSGen 12 cDNA Sequence and PSGen 12 Protein Sequence

Figure 35 B

PSGen 13 cDNA Sequence and PSGen 13 Protein Sequence

15

Figure 35 C

PEGen 28 cDNA Sequence and PEGen 28 Protein Sequence

Figure 35D

20 PEGen 32 cDNA Sequence and PEGen 32 Protein Sequence

Figure 35 E

PEGen 42 cDNA Sequence and PEGen 42 Protein Sequence

25 Figure 35 F

PEGen 45 cDNA Sequence

Figure 35 G-1 and Figure 35 G-2

30 PEGen 50 cDNA Sequence which are different parts of the gene.

Figure 36

PSGen 27 - Novel

Detailed Description of the Invention

This invention provides a method for identifying differentially expressed nucleic acids between two samples, comprising: (a) selecting a first and second
5 nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) performing reciprocal subtraction between the nucleic acid samples to produce two subtracted nucleic acid samples; (c) amplifying the two subtracted nucleic acid samples; and
10 (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

In an embodiment, the nucleic acid samples are mRNA or derived from mRNA. In another embodiment, the nucleic
15 acid samples are total RNA. In another embodiment, the nucleic acid samples are cDNA. In another embodiment, the nucleic acid samples are a nucleic acid library.

In an embodiment, differentially expressed nucleic acids
20 are expressed at different levels. In a further embodiment, one of the nucleic acids is not expressed. In a different embodiment, one of the nucleic acids is expressed in truncated form.

As used herein, reciprocal subtraction includes using
25 nucleic acid sample A to subtract common nucleic acids from nucleic acid sample B (based on hybridization) and also using nucleic acid sample B to subtract common nucleic acids from nucleic sample A. In an embodiment,
30 the complement of nucleic acid sample A is used to subtract nucleic acids from nucleic acid sample B and the complement of nucleic acid sample B is used to subtract nucleic acids from nucleic acid sample A. In a further embodiment, the RNA of nucleic acid sample A is used to
35 subtract nucleic acids from nucleic acid sample B and the RNA of nucleic acid sample B is used to subtract nucleic acids from nucleic acid sample A. In yet another

embodiment, the cDNA of nucleic acid sample A is used to subtract nucleic acids from nucleic acid sample B and the cDNA of nucleic acid sample B is used to subtract nucleic acids from nucleic acid sample A.

5

As used herein, methods of amplification include PCR and rolling circle replication.

10

A basic description of nucleic acid amplification is described in Mullis, U.S. Patent No. 4,683,202, which is incorporated herein by reference. The amplification reaction uses a template nucleic acid contained in a sample, two primer sequences and inducing agents. The extension product of one primer when hybridized to the

15

second primer becomes a template for the production of a complementary extension product and vice versa, and the process is repeated as often as is necessary to produce a detectable amount of the sequence.

20

The inducing agent may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E.coli* DNA polymerase I, thermostable *Taq* DNA polymerase, Klenow fragment of *E.coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase and other enzymes which will facilitate combination of the nucleotides in the proper manner to form amplification products. The oligonucleotide primers can

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be synthesized by automated instruments sold by a variety of manufacturers or can be commercially prepared based upon the nucleic acid sequence of this invention.

35

This invention also provides a method for identifying differentially expressed nucleic acids between two samples, comprising: a) selecting a first and second nucleic acid sample; b) producing libraries for the first

and second nucleic acid sample; c) amplifying the two libraries; d) performing reciprocal subtraction between the amplified libraries to produce two subtracted libraries; and e) comparing the two subtracted libraries to identify differentially expressed nucleic acids.

This invention also provides a method for identifying differentially expressed nucleic acids between two samples, comprising: (a) selecting a first and second nucleic acid sample, wherein the nucleic acid samples contain a repertoire of nucleic acids; (b) amplifying the two nucleic acid samples; (c) performing reciprocal subtraction between the amplified nucleic acid samples to produce two subtracted nucleic acid samples; and (d) comparing the two subtracted nucleic acid samples to identify differentially expressed nucleic acids.

This invention also provides the above-described methods, wherein the two subtracted nucleic acid samples from step c are amplified prior to the comparing of step d.

This invention also provides the above-described methods, wherein the each of the nucleic acid samples comprises a library of nucleic acids.

This invention also provides the above-described methods, wherein the nucleic acid samples are obtained from total cellular RNA purified by hybridization with oligo (dT).

This invention also provides the above-described methods, wherein the nucleic acid samples are obtained from total RNA from E11 and E11-NMT cells.

E11 is an adenovirus-transformed rat embryo cell line that acquires an aggressive oncogenic progression phenotype when injected into athymic nude mice and reisolated in cell culture (E11-NMT).

This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells in different developmental stages.

5

This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells from different tissue types.

10

This invention further provides the above-described methods, wherein the first and second nucleic acid samples are obtained from cells that differ in their exposure to external factors or in their gene expression.

15

In an embodiment, cells that differ in their exposure to external factors or in their gene expression includes any cells that may have different levels of gene expression, wherein some genes may not be expressed at all. In another embodiment, cells that differ in their exposure to external factors or in their gene expression includes any cells that are likely to have different levels of gene expression, wherein some genes may not be expressed at all. In still another embodiment, cells that differ in their exposure to external factors or in their gene expression includes any cell that has a phenotypically recognizable difference.

20
25

A short list of examples of cells that differ in their exposure to external factors or in their gene expression includes: cancerous versus normal cells, advanced cancer progression cells versus earlier cancer stage cells, diseased cells versus nondiseased cells, infected cells versus noninfected cells, later developmental stage cells versus earlier developmental stage cells, cells after DNA damage versus cells before DNA damage, senescent cells versus younger cells, cells induced by growth factors

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versus cells not induced by growth factors, cells in the process of neurodegeneration versus normal cells, and cells exposed to a chemotherapeutic agent versus normal cells.

5

As used herein, different tissues types include but are not limited to tissues containing: cells grown under or exposed to different conditions, cells in different stages of development, cells treated with agents
10 modifying cellular physiology, and cells having different functions.

In an embodiment, cells at different stages of development are cells taken or analyzed at times
15 differing by one or more hours in the development of the cell or organism.

Further, this invention provides the above-described methods, wherein the amplifying of step (d) comprises PCR
20 amplification.

Also, this invention provides the above-described methods, wherein the 3' primer used in the PCR amplification is an oligo dT 3' primer. A few examples
25 of oligo dT primers are T₁₃, T₁₃A, and T₁₃GA.

In addition, this invention provides the above-described methods, wherein the 3' primer used in the PCR amplification is a single anchor oligo dT 3' primer.
30 Oligo dT 3' primers include T₁₃A, T₁₃C, and T₁₃G.

This invention provides the above-described methods, wherein the PCR amplification uses a set of random primers.

35

This invention provides the above-described methods, wherein the 5' primer is an arbitrary primer.

This invention also provides the above-described methods, wherein the comparing of step (e) comprises using a gel to separate the nucleic acids from both of the subtracted libraries.

5

In an embodiment, the gel is a polyacrylamide gel. In another embodiment, the gel is an agarose gel.

10 This invention further provides the above-described methods, further comprising PCR amplifying the first and second nucleic acid samples.

15 This invention also provides the above-described methods, further comprising reamplifying differentially expressed bands.

This invention also provides the above-described methods, further comprising reamplifying differentially expressed nucleic acid.

20

In one method of reamplifying differentially expressed bands, differentially amplified bands from plasmids of each subtracted library were marked with an 18G needle through the film and cut out with a razor. The cut out differentially expressed bands can be reamplified (i.e. by PCR) and examined by reverse Northern and Northern blot analyses.

25

30 In addition, this invention provides the above-described methods, wherein the comparing of step (e) comprises comparing the band intensities of the two amplified differentially expressed nucleic acids.

35 In addition, this invention provides the above-described methods, wherein the nucleic acid samples are mRNA or cDNA derived from mRNA.

In addition, this invention provides the above-described methods, wherein the comparing of step (e) comprises comparing the quantities of the two amplified differentially expressed nucleic acids.

5

This invention further provides the above-described methods, wherein the differences in band intensity between the two subtracted libraries are electronically quantified.

10

This invention further provides the above-described methods, wherein the differences in the quantities of nucleic acid between the two subtracted libraries are electronically quantified.

15

In one embodiment, electronic quantification involves using a scanner to detect the bands. In a further embodiment, computer software, such as Corel Draw, can be used to determine the pixel intensity of the scanned image, thereby quantifying the band intensity.

20

Also, this invention provides the above-described methods, wherein the libraries of step (b) are constructed with λ -ZAP cDNA library kits. One skilled in the art would recognize that any cDNA library would be suitable.

25

This invention provides the isolated nucleic acid identified by the the above-described methods, wherein the nucleic acid was not previously known.

30

This invention also provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 12 (AI 144569).

35

In addition, this invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid

is the nucleic acid designated PSGen 13 (Accession No. AI 144570).

5 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 23.

10 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 24.

15 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 25.

20 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 26 (Accession No. AI 144571).

25 This invention also provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 27 (Accession No. AI 144572).

30 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 28 (AI 144573).

35 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PSGen 29 (AI 144574).

This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 13 (AI 144564).

This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 14 (AI 144565).

- 5 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 15.

10 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 24 (Accession No. AI 144566).

15 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 28 (AI 144567).

20 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 32 (AI 144568).

25 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 42.

This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 43.

30 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 44.

35 This invention provides the above-described isolated nucleic acid, wherein the isolated nucleic acid is the nucleic acid designated PEGen 48.

This invention further provides a previously unknown isolated nucleic acid molecule identified by the above-described methods which comprises (a) one of the nucleic acid sequences as set forth in Figure 35; (b) a sequence
5 being degenerated to a sequence of (a) as a result of the genetic code; (c) a sequence encoding one of the amino acid sequences as set forth in Figure 35. (d) a sequence of at least 12 nucleotides capable of specifically hybridizing to the sequence of (a), (b) or (c).

10

Finally, this invention provides a purified polypeptide comprising one of the amino acid sequence as set forth in Figure 35.

15 The sequences of the cDNA of PSGen 12, PSGen 13, PSGen 26, PSGen 27, PSGen 28, PSGen 29, PEGen 13, PEGen 14, PEGen 24, PEGen 28, and PEGen 32 were submitted to GenBank and assigned with accession numbers AI 144569, AI 144570, AI 144571, AI 144572, AI 144573, AI 144574, AI
20 144564, AI 144565, AI 144566, AI 144567 and AI 144568 respectively.

This invention will be better understood from the Experimental Details which follow. However, one skilled
25 in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

30 Experimental Details

We presently describe a reciprocal subtraction differential RNA display (RSDD) approach that efficiently and consistently reduces the complexity of DDRT-PCR and results in the identification and cloning of genes
35 displaying anticipated differential expression. Proof of principle for the RSDD approach has come from its application for the identification of genes

differentially expressed during cancer progression. RSDD has resulted in the identification and cloning of genes displaying elevated expression in progressed tumor cells (PEGen) and reduced expression in progressed tumor cells (PSGen). The model used for RSDD was an adenovirus-transformed rat embryo cell line, E11, that acquires an aggressive oncogenic progression phenotype when injected into athymic nude mice and reisolated in cell culture (E11-NMT) (10,33,34). Injection of E11 cells into nude mice results in tumors in 100% of animals with a tumor latency time of approximately 35 to 40 days, whereas E11-NMT cells form tumors in 100% of nude mice with a tumor latency time of 15 to 20 days (10,34,35). Additionally, E11 cells form colonies in agar with an efficiency of ~3%, whereas E11-NMT display an agar cloning efficiency of >30% (10,33,34). The increased tumorigenicity and enhanced anchorage independence phenotypes are key indicators of tumor progression in the E11/E11-NMT model system (10,33,34).

Differential RNA display was directly performed with reciprocally subtracted cDNA plasmid libraries (E11 minus E11-NMT and E11-NMT minus E11). Compared with the subtraction of PCR-amplified cDNA in Hakvoort et al., the subtracted cDNA libraries used in this experiment are free from potential PCR artifacts and provide more stable and consistent sources for DDRT-PCR analyzes. In addition, three single anchored oligo dT 3' primers were used instead of two-base-anchored approach described by Hakvoort et al (32). To further streamline the DDRT-PCR procedure, reamplified cDNAs identified using RSDD were analyzed using the reverse Northern blotting procedure (35,36). cDNAs displaying differential expression by reverse Northern blotting were subsequently confirmed for true differential expression by Northern analysis. These modifications incorporated in the RSDD strategy result in an efficient approach for using subtractive hybridization

and DDRT-PCR for identifying differentially expressed genes.

Methods

5 Total RNA from E11 and E11-NMT cells was isolated by the guanidinium isothiocyanate/CsCl centrifugation procedure and poly A⁺ RNA was purified with oligo(dT) cellulose chromatography (5). Two λ -ZAP cDNA libraries from E11 and E11-NMT mRNA's were constructed with λ -ZAP cDNA library
10 Kits (Stratagene) following the manufacturer's protocol. Reciprocal subtraction between E11 and E11-NMT libraries was performed and two subtracted cDNA libraries (E11 minus E11-NMT and E11-NMT minus E11) were constructed as described previously. Bacterial plasmid libraries from
15 the subtracted λ -ZAP cDNA libraries were obtained by *in vivo* excision following the manufacturer's protocol (Stratagene) and the plasmids were isolated with Qiagen columns (Qiagen Inc.).

20 The purified plasmids of reciprocally subtracted cDNA libraries were directly subjected to differential display as in Liang et. al. (38) with minor modifications. The plasmids of reciprocally subtracted cDNA libraries were PCR-amplified with the combination of three single-anchor
25 3' primers (T₁₃ A, T₁₃ C or T₁₃ G) and 18 arbitrary 5' 10-mer primers obtained from Operon Technology Inc. (Alameda, CA. OPA 1-20 except OPA1 and 3). The 20 μ l PCR reaction consisted of 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 2 μ M each dNTP, 0.2 μ M 5' arbitrary primer,
30 1 μ M 3' anchor primer, 50 ng of plasmid of a subtracted library, 10 μ Ci α -³⁵S-dATP (3000 Ci/mmol from Amersham) and 1 U of Taq DNA polymerase (Gibco BRL). The parameters of PCR were 30 sec at 95 C, 40 cycle of 30 sec at 95 C, 2 min. at 40 C and 30 sec at 72 C and additional
35 5 min. at 72 C. After the cycling, 10 μ l of 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol were added to each PCR reaction. The mixture was heated

at 95 °C for 2 min and separated in a 5% denaturing DNA sequencing gel maintained at 50 °C. PCR reactions of plasmids from each subtracted library in a primer set were run side by side. Differentially amplified bands from plasmids of each subtracted library were marked with an 18G needle through the film and cut out with a razor. The gel slice was put in 100 µl TE pH 8.0 and incubated at 4 °C overnight. After the incubation, the mixture was boiled for 5 min and microcentrifuged for two min. The supernatant was collected and stored at -20 °C until reamplification. The band extract was reamplified with the same cycling parameters in a 50 µl reaction consisting of 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 20 µM each dNTP, 0.2 µM 5' arbitrary primer, 1 µM 3' anchor primer, 5 µl of band extract and 2.5 U of Taq DNA polymerase (Gibco BRL).

Differential expression of the reamplified DNA fragment was scrutinized by reverse Northern and Northern blot analyses. In reverse Northern analysis, after confirmation in a 1% agarose gel, the reamplified DNA fragment (10 µl of PCR reaction) was mixed with 90 µl TE and spotted on a positively charged Nylon membrane (Boehringer Mannheim) with a 96-well vacuum manifold. The membrane was soaked with denaturing and neutralizing solution successively, and the spotted DNA was crosslinked to the membrane with a UV crosslinker (Stratagene). ³²P-labeled first strand cDNA was prepared by reverse transcription of total RNA. After heating at 70 °C for 10 min and quenching on ice for two min, 0.4 µM each T₁₃A, T₁₃G and T₁₃C and 10 µg total RNA mixture was added with 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.02 mM dCTP, 0.5 µl RNase inhibitor (Gibco BRL), 100 µCi dCTP (3000 Ci/mmole from Amersham) and 200 U Superscript RT II (Gibco BRL) in a final 25 µl reaction. The reaction mixture was incubated at 42 °C for one hr and at

37 °C for 30 min after addition of 2 μ l of RNase H (10U, Gibco BRL). The membrane was hybridized at 42 °C overnight in a 50% formamide hybridization solution. The hybridized membrane was washed at room temperature for 15 min with 2X SSC containing 0.1% SDS twice and at 55 °C for at least one hr with 0.1X SSC containing 0.1% SDS, successively. The membrane was probed with the ³²P-labeled cDNA of E11, stripped off and probed with ³²P-labeled cDNA of E11-NMT. The signal intensity of each spot was normalized against that of GAPDH and compared between E11 and E11-NMT. Reamplified DNA fragments displaying differential expression levels \geq 1.8-fold higher between the two cell types were selected and analyzed by Northern blotting analysis.

In Northern blot analysis, 10 μ g of total RNA from E11 and E11-NMT cells were run side-by-side in a 1% agarose gel with formaldehyde and transferred to a positively charged Nylon membrane. Reamplification reaction (5 μ l) was ³²P-labeled with a multiprime labeling kit (Boehringer Mannheim) used to probe the membrane as described above. DNA fragments expressed differentially between E11 and E11-NMT in Northern blot analyses were cloned into the Eco RV site of the pZero-2.1 cloning vector (Invitrogene) and sequenced. In order to confirm differential expression, the cloned cDNA fragment was released by Eco RI -Xho I, ³²P-labeled and used to probe Northern blots as described above. Samples of RNAs from various E11 and E11-NMT derivatives displaying either a progressed or suppressed progression phenotype, based on nude mice tumorigenesis and soft agar cloning assays were analyzed. These included E11, E11-NMT, CREF X E11-NMT F1 and F2 somatic cell hybrids (suppressed progression phenotype), CREF X E11-NMT R1 and R2 somatic cell hybrids (progression phenotype), E11 X E11-NMT A6 somatic cell hybrid (suppressed progression phenotype), E11 X E11-NMT A6TD tumor-derived somatic cell hybrid (progression

phenotype), E11 X E11-NMT 3b somatic cell hybrid (suppressed progression phenotype), E11 X E11-NMT 2a (progression phenotype), E11-NMT AZA B1 and C1 5-azacytidine treated E11-NMT clones (suppressed progression phenotype), E11-ras R12 clone containing the Ha-ras oncogene (progression phenotype) and E11-HPV E6/E7 clone containing the human papilloma virus-18 E6 and E7 gene region (progression phenotype). Differential expression of the PEGen and PSGen genes in the various cell types was confirmed using ³²P-labeled probes and Northern hybridization analysis. After reconfirmation of differential expression, the plasmids containing the differentially expressed DNA fragments were sequenced by the dideoxy sequencing procedure.

15

Results and Discussion

Subtraction hybridization provides a direct means of enriching for unique cDNA species and eliminating common sequences between complex genomes. DDRT-PCR is a proven methodology for the rapid identification and cloning of differentially expressed sequences between cell types (3,4,22). In principle, subtraction hybridization combined with DDRT-PCR should reduce band complexity which often obscures the identification of differentially expressed genes and generates false positive signals (23,28). This strategy, RSDD, has been used to analyze genes differentially expressed during transformation progression. The differential RNA display pattern of E11 and E11-NMT cells using standard differential RNA display DDRT-PCR) and RSDD is shown in Fig. 1 (Left Panel). As predicted, the differential RNA display pattern of RSDD was much less complex than that of DDRT-PCR. The majority of bands common to both cDNA samples were eliminated using RSDD. These experiments demonstrate that subtractive hybridization prior to differential RNA display is effective in simplifying display patterns

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permits the efficient identification of differentially expressed cDNAs. Since RSDD significantly reduced the number of bands displayed, single anchor oligo dT primers, that can increase band numbers, were successfully used in subsequent applications of the RSDD approach (Fig. 1; Right Panel). Using RSDD, 235 differentially displayed cDNAs in the E11/E11-NMT tumor progression model system were isolated.

10 Hakvoort et al. (32) used a reciprocal subtraction approach to analyze gene expression changes resulting during liver regeneration following 70% hepatectomy, i.e., normal liver subtracted from partially hepatectomized regenerating liver and vice versa. 15 Although some bands displayed apparent enrichment, the complexity of the display pattern did not show appreciable simplification. These results are in stark contrast to RSDD, which results in a clear delineation and simplification of differentially expressed amplified 20 bands (Figs. 1). Although conceptually similar, RSDD is significantly more effective than the subtraction plus DDRT-PCR approach described by Hakvoort et al. (32). The improved efficiency of RSDD versus the Hakvoort et al. (32) approach can be attributed to several factors. The 25 approach of Hakvoort et al. (32) is based on the subtraction procedure described by Wang and Brown (38). This approach involves multiple rounds of PCR-amplification prior to each round of subtractive hybridization. In contrast, RSDD involves a single round of reciprocal subtraction that does not involve PCR 30 amplification (5,10). In this respect, the complicated display pattern observed by Hakvoort et al. (32) even after three or four rounds of subtraction might result from reduced subtraction efficiency, PCR artifacts or a combination of these problems. Increasing the number of 35 reactions by using two-base pair anchored oligo dT primers did not reduce the complexity of displayed bands

(32). In these contexts, a critical component for the successful use of RSDD involves the use of an appropriate subtraction hybridization protocol, that can efficiently reduce cDNA complexity and generate stable populations of cDNAs for analysis.

Previous studies demonstrate that different gene cloning strategies, including DDRT-PCR, subtraction hybridization and electronic display, identify dissimilar differentially expressed genes (18). These results suggest that a single approach for gene identification may not identify the complete spectrum of differentially expressed genes (18). Similarly, RSDD and DDRT-PCR do not resolve the same differentially expressed bands (Fig. 1). Unique bands identified in DDRT-PCR that were differentially expressed when analyzed by Northern blotting were not the same as those found using RSDD and vice versa. These results are not surprising, since, as indicated above, subtraction hybridization and differential RNA display identified distinct differentially expressed genes. Apparently, specific differentially expressed genes are lost during subtraction hybridization and differential RNA display of subtracted cDNAs. On the basis of these considerations, it will be essential to use multiple gene discovery approaches to identify and clone the complete spectrum of differentially expressed genes.

DDRT-PCR can generate large numbers of differentially displayed bands making subsequent analysis both labor intensive and a daunting challenge. In order to reduce these limitations of DDRT-PCR, RSDD has been used in combination with reverse Northern analyses of isolated cDNAs. Gel extracted cDNA fragments were reamplified, dot-blotted on Nylon membranes and successively probed with reverse transcribed ³²P-cDNA from E11 or E11-NMT RNAs (Fig. 2). Signals were detected in 181 reamplified bands

5 out of 235 (77%). This number is lower than that observed using DDRT-PCR (51 out of 54). However, this comparison may not be accurate since only four arbitrary primers were used for DDRT-PCR and fewer differentially expressed bands were detected and isolated. A possible reason for the high incidence of false positives in RSDD may be due to the existence of foreign plasmid-like DNA in the cDNAs and the inaccurate reading properties of DDRT-PCR.

Table 1. Differentially Expressed cDNA Fragments
Cloned by DDRT-PCR.

	Nomenclature	Identity	Homology
5	PEGen 41	To be determined	
	PEGen 42	Novel	Novel
10	PEGen 43	Novel	Novel
	PEGen 44	Novel	Novel
	PEGen 45	Hoxa11 locus antisense	mouse 90%
15	PEGen 46	Glutamyl t-RNA synthetase	human 59%
	PEGen 48	Novel	Novel
	PEGen 50	Novel	Novel
20	PSGen 1	Supervillin	B. <i>taurus</i> 80%
	PSGen 2	HTLV-1 Tax interacting protein	human 91%
	PSGen 4	Proteasome activator	Rat 100%
25	PSGen 27	Novel	

30 The signal intensities of the various cDNAs in reverse Northern analysis were quantified and normalized against that of GAPDH, which remained unchanged in E11 and E11-NMT cells. The PEG-3 (PEGen-3) gene (10) was used as an additional control, to verify increased expression in E11-NMT versus E11 cells. In the reverse Northern

analyses, PEGen-3 levels were 4-fold higher in E11-NMT than in E11 cells, which coincided with Northern blotting results, thereby demonstrating the concordance of reverse Northern and Northern assays. A ≥ 1.8 -fold differential cut-off (after normalization for GAPDH expression) was used to identify and isolate cDNA bands displaying modified expression in E11 versus E11-NMT cells. This resulted in the identification of 7 cDNAs with higher expression in E11 versus E11-NMT cells and 65 cDNAs with elevated expression in E11-NMT versus E11 cells. These results suggest that tumor progression in E11-NMT cells correlates with the increased expression of a large number of genes, whereas only a smaller subset of genes display decreased expression.

A problem present in DDRT-PCR, that is reduced but still can occur in RSDD, is the isolation of multiple cDNA species from what appears to be a single amplified band. When this occurs, these multiple species can produce spurious results when analyzed by reverse Northern analyses. For example, if two distinct species are isolated, one displaying modified expression and a second not displaying modified expression, an accurate estimate of differential expression will not be obtained by reverse Northern analysis. In this case, a number of potential false positives generated using reverse Northern analyses, may in reality not be false positives, but instead may represent multiple cDNAs. This problem may be ameliorated by performing single strand conformational polymorphism (SSCP) or reverse Northern analyses using cloned cDNA populations (39,40).

The expression pattern of representative RSDD-derived cDNAs in E11 versus E11-NMT and in a more expanded E11/E11-NMT progression cell culture series is shown in Figs. 3 and 4, respectively. Reverse Northern results correlated well with Northern blots using E11 and

E11-NMT (~80% concordance) or a larger panel of cells differentially displaying the progression phenotype, including progression negative, E11, CREF x E11-NMT F1, CREF X E11-NMT F2, E11 X E11-NMT A6, E11 X E11-NMT 3b, 5 E11-NMT Aza B1 and E11-NMT Aza C1, and progression positive E11-NMT, CREF X E11-NMT R1, CREF X E11-NMT R2, E11 X E11-NMT A6TD, E11 X E11-NMT IIa, E11-ras and E11-HPV E6/E7. Sequence analysis of the various progression upregulated genes (PEGen) and progression 10 suppressed genes (PSGen) identified both known and unknown genes (Table 2). Known PEGen genes included PEGen 7 (HPV16 E1BP), PEGen 8 (PFK-C), PEGen 21 (FIN 14) and PEGen 26 (poly ADP-ribose polymerase) and a known PSGen gene was PSGen 10 (ferritin heavy chain). Two 15 PEGen genes out of six were found to be novel (PEGen 14 and PEGen 24) and two PSGen genes out of three were found to be novel (PSGen 12 and PSGen 13) (Table 2).

Table 2. Differentially Expressed cDNA Fragments
Cloned by RSDD

5	<hr/>		
	Nomenclature	Identity	Homology
<hr/>			
	PEGen 7	HPV16 E1BP	Human 90%
	PEGen 8	PFK-C	Rat 100%
10	PEGen 13	Novel	Novel
	PEGen 14	Novel	Novel
	PEGen 15	Novel	Novel
15	PEGen 21	FIN 14	Mouse 94%
	PEGen 24	Novel	Novel
20	PEGen 26	Poly ADP-ribose Polymerase	Rat 100%
	PEGen 28	Novel	Novel
	PEGen 32	Novel	Novel
25	PSGen 10	Ferritin Heavy Chain	Rat 100%
	PSGen 12	Novel	Novel
30	PSGen 13	Novel	Novel
	PSGen 23	Novel	Novel

	PSGen 24	Novel	Novel
	PSGen 25	Novel	Novel
5	PSGen 26	Novel	Novel
	PSGen 27	Novel	Novel
10	PSGen 28	Novel	Novel
	PSGen 29	Novel	Novel

15 PEGen 7 is expressed at ~ 5-fold higher levels in E11-NMT than in E11 cells. PEGen 7 is ~90% homologous to 16E1-BP, a cDNA encoding a protein identified using the yeast two-hybrid assay that interacts with human papillomavirus type 16 E1 protein (41). 16E1-BP encodes a 432aa protein of unknown function but does contain an ATPase signature motif (Gly-X₄-Gly consensus ATP binding motif at aa 179 through 186). 16E1-BP appears to be a form of TRIP13, a protein previously shown to bind thyroid hormone receptor in yeast two-hybrid assays. The role of PEGen 7/16E1-BP in the progression phenotype in the E11/E11-NMT progression model is not known. Additional studies are necessary to determine if this gene change is associative or causative of transformation progression.

30 PEGen 8 is expressed at ~3- to 4- fold higher levels in E11-NMT than in E11 cells. PEGen 8 shows 100% homology to rat phosphofructokinase C (PFK-C) (42). PFK catalyzes the rate-limiting and committed step in glycolysis, the conversion of fructose 6-phosphate to fructose 1,6-biphosphate. Three subunit isozymes of PFK have been identified, that form homo- and heterotetramers with differing catalytic and allosteric properties. PFK-M is

specific for cardiac and skeletal muscle, PFK-L is expressed in many tissues but is most abundant in the liver and PFK-C is expressed in several brain regions and the anterior pituitary but not in liver, skeletal muscle, or several other human tissues. The cDNA of PFK-C isolated from a rat hypothalamic cDNA library is 2643 bp and encodes a protein of 765aa (42). In a recent study, Sanchez-Martinez and Aragon (43) demonstrated that PFK-C is the predominant form of PFK in ascites tumor cells (obtained from a transplantable mouse carcinoma of mammary origin), whereas PFK-L is most abundant in the normal mammary gland. These results suggest the interesting possibility that PFK-C might contribute to the malignant nature of specific target cells. The role of PEGen 8/PFK-C in progression in the E11/E11-NMT model remains to be determined.

PEGen 21 is expressed at ~3- to 4-fold higher levels in E11-NMT than in E11 cells. PEGen 21 displays ~94% homology with the fibroblast growth factor-4 inducible gene FIN-14 (44). FIN-14 is a novel cDNA of unknown function that hybridizes with a 4.5 kb mRNA that is induced 4-fold in NIH3T3 mouse cells following treatment with FGF-4. The induction of FIN-14 occurs late (18 hr) after treatment with FGF-4 and does not occur when cells are treated for 18 hr with FGF-4 in the presence of cycloheximide (44). These results confirm that FIN-14 encodes a late-inducible gene. Moreover, nuclear run-on assays document that FIN-14 is transcriptionally activated in NIH3T3 cells following growth factor stimulation. Tissue distribution studies indicate expression of a single mRNA species in the kidney with low levels of expression observed in several other tissues including testis and thymus. Mouse embryogenesis studies indicate that FIN-14 expression occurs constitutively in mouse embryos between day 10.5 and 15.5. Unlike NIH3T3, FIN-14 was constitutively expressed in PC12 cells and its level

did not vary appreciably in response to growth factor stimulation. The role of PEGen 21/FIN-14 in progression in E11/E11-NMT model system is not currently known.

5 The PGen cDNAs, PGen-12 and PGen-13, consist of sequences without homology to those presently reported in various DNA databases. Expression of these cDNAs is ~3- to 4-fold higher in E11 versus E11-NMT cells (Fig. 3). It is not currently known whether these genes simply
10 correlate with or functionally regulate the progression phenotype. The identification of full-length cDNAs for PGen-12 and PGen-13 are in progress and once identified experiments can be conducted to directly define the role of these PGen's in cancer progression.

15 We presently demonstrate that a modified differential RNA display technique, RSDD, can efficiently identify differentially expressed cDNAs. As predicted, subtractive hybridization prior to differential RNA
20 display greatly reduces band complexity, a problem encountered in standard DDRT-PCR in which RNA samples are directly analyzed without subtraction. Unlike a previous report using subtracted cDNAs processed through successive rounds of PCR (32,45), common bands were
25 eliminated using reciprocally subtracted cDNA libraries that had not been processed using PCR. In addition to subtraction hybridization, the discovery of differentially expressed genes was further streamlined by using reverse Northern analyses with isolated cDNAs.
30 With 3 single anchored oligo dT primers and 18 arbitrary 5' primers, 72 bands were identified that displayed differential expression using reverse Northern analysis. Currently, 40 of these cDNA species have been analyzed by Northern blotting and found to display differential
35 expression in E11 versus E11-NMT cells. Subsequent studies with the majority of these RSDD cDNAs demonstrated coordinated expression with the progression

phenotype in a large panel of unprogressed and progressed transformed cells. Current sequence analysis of the cloned cDNA fragments revealed 9 different genes, including 4 novel genes not reported in recent DNA
5 databases. RSDD represents a method of choice either as a more efficient and less time consuming modification of the differential RNA display strategy or as a screening methodology for identifying differentially expressed genes in reciprocally subtracted cDNA libraries.

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Second Series of Experiments

Presently described is a RSDD approach that efficiently and consistently reduces the complexity of DDRT-PCR and results in the identification and cloning of genes displaying anticipated differential expression. The model used for RSDD was an adenovirus-transformed rat embryo cell line, E11, that acquires an aggressive oncogenic progression phenotype when injected into athymic nude mice and reestablished in cell culture (E11-NMT) (6,26,27). Injection of E11 cells into nude mice results in tumors in 100% of animals with a tumor latency time of approximately 35 to 40 days, whereas E11-NMT cells form tumors in 100% of nude mice with a tumor latency time of 15 to 20 days (6,26,27). Additionally, E11 cells form colonies in agar with an efficiency of ~3 %, whereas E11-NMT display an agar cloning efficiency of >30% (6,26,27). The increased tumorigenicity and enhanced anchorage independence phenotypes are key indicators of tumor progression in the E11/E11-NMT model system (6,26,27). RSDD has resulted in the identification and cloning of genes displaying elevated expression in progressed tumor cells (progression elevated gene, PEGen) and suppressed expression in progressed tumor cells (progression suppressed gene, PSGen).

MATERIALS AND METHODS

RNA isolation and cDNA library construction. Total RNA from E11 and E11-NMT cells was isolated by the guanidinium isothiocyanate/CsCl centrifugation procedure and poly(A)⁺ RNA was purified with oligo(dT) cellulose chromatography(5). Two λ -ZAP cDNA libraries from E11 and E11-NMT mRNAs were constructed with λ -ZAP cDNA library kits (Stratagene) following the manufacturer's protocol. Reciprocal subtraction between E11 and E11-NMT libraries was performed and two subtracted cDNA libraries (E11 minus E11-NMT and E11-NMT minus E11) were constructed as

described(5,6). Plasmid cDNA libraries from the subtracted λ -ZAP cDNA libraries were obtained by in vivo excision following the manufacturer's protocol (Stratagene) and the plasmids were isolated with Qiagen columns (Qiagen, Chatsworth, CA.).

RSDD methodology. The purified plasmids of reciprocally subtracted cDNA libraries were directly subjected to differential display as in Liang et al. (28) with minor modifications. The plasmids of reciprocally subtracted cDNA libraries were PCR-amplified with the combination of three single-anchor 3' primers ($T_{13}A$, $T_{13}C$ or $T_{13}G$) and 18 arbitrary 5' 10-mer primers obtained from Operon Technology Inc. (Alameda, CA. OPA 1-20 except OPA1 and 3). The 20 μ l PCR reaction consisted of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2 μ M each dNTP, 0.2 μ M 5' arbitrary primer, 1 μ M 3' anchor primer, 50 ng of plasmid of a subtracted library, 10 μ Ci α - ^{35}S -dATP (3,000 Ci/mmol from Amersham) and 1 unit of Taq DNA polymerase (Gibco/BRL). The parameters of PCR were 30 sec at 95°C, 40 cycles of 30 sec at 95°C, 2 min at 40°C and 30 sec at 72°C and additional 5 min. at 72°C. After the cycling, 10 μ l of 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol were added to each PCR reaction. The mixture was heated at 95°C for 2 min and separated in a 5% denaturing DNA sequencing gel maintained at 50°C. PCR reactions of plasmids from each subtracted library in a primer set were run side by side. Differentially amplified bands from plasmids of each subtracted library were marked with 18G needle through the film and cut out with a razor. The gel slice was put in 100 μ l TE (pH 8.0) and incubated at 4°C overnight. After the incubation, the mixture was boiled for 5 min and microcentrifuged for two min. The supernatant was collected and stored at -20°C until reamplification. The band extract was reamplified with the same cycling parameters in a 50 μ l reaction consisting of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM

MgCl₂, 20 μM each dNTP, 0.2 μM 5' arbitrary primer, 1 μM 3' anchor primer, 5 μl of band extract and 2.5 units of Taq DNA polymerase (Gibco/BRL).

5 **Reverse Northern Blotting Procedure.** Differential expression of the reamplified DNA fragment was scrutinized by reverse Northern and Northern blot analyses. In reverse Northern analysis, after confirmation in a 1% agarose gel, the reamplified DNA
10 fragment (10 μl of PCR reaction) was mixed with 90 μl TE and spotted on a positively charged Nylon membrane (Boehringer Mannheim) with a 96-well vacuum manifold. The membrane was soaked with denaturing and neutralizing solution successively, and the spotted DNA was
15 crosslinked to the membrane with a UV crosslinker (Stratagene). ³²P-labeled first strand cDNA was prepared by reverse transcription of total RNA. After heating at 70°C for 10 min and quenching on ice for two min, 0.4 μM each T₁₃A, T₁₃G and T₃C and 10 μg total RNA mixture was
20 added with 50 mM Tris-HCl, (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.02 mM dCTP, 0.5 μl RNase inhibitor (Gibco/BRL), 100 μCi dCTP (3,000 Ci/mmol from Amersham) and 200 units Superscript RT II (Gibco/BRL) in a final 25 μl reaction.
25 The reaction mixture was incubated at 42°C for one hour and at 37°C for 30 min after addition of 2 μl of RNase H (10 units, Gibco/BRL). The membrane was hybridized at 42°C overnight in a 50% formamide hybridization solution. The hybridized membrane was washed at room temperature for 15
30 min with 2X standard saline citrate containing 0.1% SDS twice and at 55°C for at least one hour with 0.1X Standard Saline Citrate containing 0.1% SDS, successively. The membrane was probed with the ³²P-labeled cDNA of E11, stripped off and probed with ³²P-labeled cDNA of E11-NMT.
35 The signal intensity of each spot was normalized against that of glyceraldehyde-3-phosphate dehydrogenase and compared between E11 and E11-NMT. Reamplified DNA

fragments displaying differential expression levels ≥ 1.8 -fold higher between the two cell types were selected and analyzed by Northern blotting analysis.

5 **Northern Blotting Analysis.** In Northern blot analysis, 10 μ g of total RNA from E11 and E11-NMT cells were run side-by-side in a 1% agarose gel with formaldehyde and transferred to a positively charged Nylon membrane. Reamplification reaction (5 μ l) was 32 P-labeled with a
10 multiprime labeling kit (Boehringer Mannheim) used to probe the membrane as described above. DNA fragments expressed differentially between E11 and E11-NMT in Northern blot analyses were cloned into the EcoRV site of the pZEro-2.1 cloning vector (Invitrogene) and sequenced.
15
To confirm differential expression, the cloned cDNA fragment was released by EcoRI-XhoI, 32 P-labeled and used to probe Northern blots as described above. Samples of RNAs from various E11 and E11-NMT derivatives displaying
20 either a progressed or suppressed progression phenotype, based on nude mice tumorigenesis and soft agar cloning assays were analyzed. These included E11, E11-NMT, CREF x E11-NMT F1 and F2 somatic cell hybrids (suppressed progression phenotype), CREF x E11-NMT R1 and R2 somatic
25 cell hybrids (progression phenotype), E11 x E11-NMT A6 somatic cell hybrid (suppressed progression phenotype), E11 x E11-NMT A6TD tumor-derived somatic cell hybrid (progression phenotype), E11 x E11-NMT 3b somatic cell hybrid (suppressed progression phenotype), E11 x E11-NMT
30 IIa (progression phenotype), E11-NMT AZA B1 and C1 5-azacytidine treated E11-NMT clones (suppressed progression phenotype), E11-Ras R12 clone containing the Ha-ras oncogene (progression phenotype) and E11-HPV E6/E7 clone containing the human papilloma virus-18 E6 and E7
35 gene region (progression phenotype). Differential expression of the PEGen and PSGen genes in the various cell types was confirmed using 32 P-labeled probes and

northern hybridization analysis. After reconfirmation of differential expression, the plasmids containing the differentially expressed DNA fragments were sequenced by the dideoxy sequencing procedure.

5

RESULTS AND DISCUSSION

Subtraction hybridization provides a direct means of enriching for unique cDNA species and eliminating common sequences between complex genomes(7,18). DDRT-PCR is a proven methodology for the rapid identification and cloning of differentially expressed sequences between cell types (1,2,28). In principle, subtraction hybridization combined with DDRT-PCR should reduce band complexity which often obscures the identification of differentially expressed genes and generates false positive signals (21,29). RSDD has been used to analyze genes differentially expressed during transformation progression (Fig. 28). Differential RNA display was directly performed with reciprocally subtracted cDNA plasmid libraries (E11 minus E11-NMT and E11-NMT minus E11) that had not been subjected to PCR. Three single anchored oligo dT 3' primers were used for subsequent amplification prior to display. To further streamline the DDRT-PCR procedure, reamplified cDNAs identified using RSDD were analyzed using the reverse Northern blotting procedure (30,31). cDNAs displaying differential expression by reverse Northern blotting were subsequently confirmed for true differential expression by Northern analysis.

30

The differential RNA display pattern of E11 and E11-NMT cells using standard differential RNA display (DDRT-PCR) and RSDD is shown in Fig. 1 (Left Panel). The differential RNA display pattern of RSDD is much less complex than that of DDRT-PCR. These experiments demonstrate that subtractive hybridization prior to differential RNA display is effective in simplifying

35

display patterns permitting the efficient identification of differentially expressed cDNAs. Since RSDD significantly reduced the number of bands displayed, single anchor oligo dT primers, that can increase band numbers, were successfully used in subsequent applications of the RSDD approach (Fig. 1; Right Panel). Using RSDD, 234 differentially displayed cDNAs in the E11/E11-NMT tumor progression model system were isolated. Hakvoort et al.(25) used a reciprocal subtraction approach to analyze gene expression changes resulting during liver regeneration following 70% hepatectomy, i.e., normal liver subtracted from partially hepatectomized regenerating liver and vice versa. Although some bands displayed apparent enrichment, the complexity of the display pattern did not show appreciable simplification. In contrast, RSDD results in a clearer delineation and simplification of differentially expressed amplified bands (Figs. 1). Although conceptually similar, RSDD is significantly more effective than the subtraction plus DDRT-PCR approach described by Hakvoort et al. (25). The reasons for the improved efficiency of RSDD versus the Hakvoort et al. (25) approach are not known. One possibility is that the differences between the experimental approaches may reflect the subtraction hybridization strategies employed. The approach of Hakvoort et al. (25) is based on the subtraction procedure described by Wang and Brown (32). This approach uses multiple rounds of PCR-amplification prior to each round of subtractive hybridization. In contrast, RSDD involves a single round of reciprocal subtraction without intermediate amplification(5,6). In this respect, the complicated display pattern observed by Hakvoort et al. (25) even after three or four rounds of subtraction might result from reduced subtraction efficiency, PCR artifacts or a combination of these problems. Increasing the number of reactions by using two-base pair anchored oligo dT

primers did not reduce the complexity of displayed bands (25). In these contexts, a critical component for the successful use of RSDD involves the use of an appropriate subtraction hybridization protocol, which can efficiently reduce cDNA complexity and generate stable populations of cDNAs for analysis.

Previous studies demonstrate that different gene cloning strategies, including DDRT-PCR, subtraction hybridization and electronic display, identify distinct subsets of differentially expressed genes (18). These results suggest that a single approach for gene identification may not identify the complete spectrum of differentially expressed genes. Similarly, RSDD and DDRT-PCR do not resolve the same differentially expressed bands (Fig. 1). Unique bands identified in DDRT-PCR that were differentially expressed when analyzed by Northern blotting were not the same as those found using RSDD and vice versa (data not shown). These results are not surprising, since, as indicated above, subtraction hybridization and differential RNA display identified distinct differentially expressed genes (18). Apparently, specific differentially expressed genes are lost during subtraction hybridization and differential RNA display of subtracted cDNAs. On the basis of these considerations, it will be essential to use multiple gene discovery approaches to identify and clone the complete spectrum of differentially expressed genes.

DDRT-PCR can generate large numbers of differentially displayed bands making subsequent analysis both labor intensive and a daunting challenge. In order to reduce these limitations of DDRT-PCR, RSDD has been used in combination with reverse Northern analyses of isolated cDNAs. Gel extracted cDNA fragments were reamplified, dot-blotted on Nylon membranes and successively probed with reverse transcribed ³²P-cDNA from E11 or E11-NMT RNAs

(Fig. 2). Signals were detected in 181 reamplified bands out of 234 (77%).

5 The signal intensities of the various cDNAs in reverse Northern analysis were quantified and normalized against that of GAPDH, which remained unchanged in E11 and E11-NMT cells. Progression elevated gene-3 (PEG-3)(6) was used as an additional control, to verify increased expression in E11-NMT versus E11 cells. In the reverse
10 Northern analyses, PEG-3 levels were 4-fold higher in E11-NMT than in E11 cells, which coincided with Northern blotting results, thereby demonstrating the concordance of reverse Northern and Northern assays. A ≥ 1.8 -fold differential cut-off (after normalization for GAPDH
15 expression) was used to identify and isolate cDNA bands displaying modified expression in E11 versus E11-NMT cells. This resulted in the identification of 7 cDNAs with higher expression in E11 versus E11-NMT cells and 65 cDNAs with elevated expression in E11-NMT versus E11
20 cells. These results suggest that tumor progression in E11-NMT cells correlates with increased expression of a large number of genes, whereas only a smaller subset of genes display decreased expression.

25 A problem frequently encountered in DDRT-PCR, that is reduced but still can occur in RSDD, is the isolation of multiple cDNA species from what appears to be a single amplified band. When this occurs, these multiple species can produce spurious results when analyzed by reverse
30 Northern analyses. For example, if two distinct species are isolated, one displaying modified expression and a second not displaying modified expression, an accurate estimate of differential expression will not be obtained by reverse Northern analysis. In this case, a number of
35 potential false positives generated using reverse Northern analyses, may in reality not be false positives, but instead may represent multiple cDNAs. By performing

single strand conformational polymorphism (SSCP) or reverse Northern analyses using cloned cDNA populations (33,34) this problem can be ameliorated.

5 The expression pattern of representative RSDD-derived cDNAs in E11 versus E11-NMT and in a more expanded E11/E11-NMT progression cell culture series is shown in Figs. 29 and 30, respectively. Reverse Northern results correlated well with Northern blots using E11 and E11-NMT
10 (~75% concordance) or a larger panel of cells differentially displaying the progression phenotype, including progression negative E11, CREF x E11-NMT F1 and F2, E11 x E11-NMT A6, E11 x E11-NMT 3b, E11-NMT Aza B1 and Aza C1 cells, and progression positive E11-NMT, CREF
15 x E11-NMT R1 and R2, E11 x E11-NMT A6TD, E11 x E11-NMT IIa, E11-Ras R12 and E11-HPV E6/E7 cells. Sequence analysis of the various PEGen cDNAs identified both unknown and known genes (Table 3). Five of 10 PEGen cDNAs (50%) were classified as novel sequences since no matches
20 were found in current DNA databases. Novel PEGen cDNAs include, PEGen 13, 14, 24, 28 and 32. Known PEGen genes included PEGen 7 (human papilloma virus-16 early region 1 binding protein; HPV16 E1BP), PEGen 8 (phosphofructokinase kinase C; PFK-C), PEGen 21 (a
25 fibroblast growth factor-4 inducible gene; FIN 14), PEGen 26 (poly ADP-ribose polymerase) and PEGen 30 (rat espl homology). In the case of the PSGen cDNAs, six of six (100%) were novel, including PSGen 12, 13, 26, 27, 28 and
30 29 (Table 3).

Table 3. PEGen and PSGen genes isolated using RSDD

	Nomenclature ^a (%) ^c	Identity ^b	Homology
	PEGen 7	Human HPV16 E1BP	90
5	PEGen 8	Rat phospho- fructokinase C (PFK-C)	100
	PEGen 13	Unknown	Novel
	PEGen 14	Unknown	Novel
	PEGen 21	Murine FIN 14	94
10	PEGen 24	Unknown	Novel
	PEGen 26	Rat poly ADP-ribose polymerase	100
	PEGen 28	Unknown	Novel
	PEGen 30	Rat espl	98
15	PEGen 32	Novel	Novel
	PSGen 12	Unknown	Novel
	PSGen 13	Unknown	Novel
	PSGen 26	Unknown	Novel
	PSGen 27	Unknown	Novel
20	PSGen 28	Unknown	Novel
	PSGen 29	Unknown	Novel

25 ^aPEGen are progression elevated genes that display elevated expression in E11-NMT versus E11 cells. PSGen are progression suppressed genes that display elevated expression in E11 versus E11-NMT cells.

30 ^bSequences have compared with reported genes in various DNA data bases (including GenBank and EMBL) and identification with known genes are indicated. Genes without homology to currently reported genes are indicated as unknown.

^cpercentage homology with known sequences, either human, rat or mouse is indicated.

Where no homology exists the cDNA is considered novel.

PEGen 7 is expressed at ~ 4-fold higher levels in E11-NMT than in E11 cells. PEGen 7 is ~98% homologous to 16E1-BP, a cDNA encoding a protein identified using the yeast
5 two-hybrid assay that interacts with human papillomavirus type 16 E1 protein (35). 16E1-BP encodes a 432aa protein of unknown function but does contain an ATPase signature motif (Gly-X4-Gly consensus ATP binding motif at aa 179 through 186). 16E1-BP appears to be a form of TRIP13, a
10 protein previously shown to bind thyroid hormone receptor in yeast two-hybrid assays. The role of PEGen 7/16E1-BP in the progression phenotype in the E11/E11-NMT progression model is not known. Additional studies are necessary to determine if this gene change is associative
15 or causative of transformation progression.

PEGen 8 is expressed at ~3- to 4- fold higher levels in E11-NMT than in E11 cells. PEGen 8 shows 100% homology to rat phosphofructokinase C (PFK-C) (36). PFK catalyzes the
20 rate-limiting and committed step in glycolysis, the conversion of fructose 6-phosphate to fructose 1,6-biphosphate. Three subunit isozymes of PFK have been identified, that form homo- and heterotetramers with differing catalytic and allosteric properties. PFK-M is
25 specific for cardiac and skeletal muscle, PFK-L is expressed in many tissues but is most abundant in the liver and PFK-C is expressed in several brain regions and the anterior pituitary but not in liver, skeletal muscle, or several other human tissues. The cDNA of PFK-C
30 isolated from a rat hypothalamic cDNA library is 2643 bp and encodes a protein of 765aa (-36). In a recent study Sanchez-Martinez and Aragon (37), demonstrated that PFK-C is the predominant form of PFK in ascites tumor cells (obtained from a transplantable mouse carcinoma of
35 mammary origin), whereas PFK-L is most abundant in the normal mammary gland. These results suggest the interesting possibility that PFK-C might contribute to the malignant nature of specific target cells. The role

presently reported of PEGen 8/PFK-C in progression in the E11/E11-NMT model remains to be determined.

5 PEGen 21 is expressed at ~3- to 4-fold higher levels in E11-NMT than in E11 cells. PEGen 21 displays ~98% homology with the fibroblast growth factor-4 inducible gene FIN-14 (38). FIN-14 is a novel cDNA of unknown function that hybridizes with a 4.5 kb mRNA that is induced 4-fold in NIH 3T3 mouse cells following treatment with FGF-4. The induction of FIN-14 occurs late (18 hr) after treatment with FGF-4 and does not occur when cells are treated for 18 hr with FGF-4 in the presence of cycloheximide (38). These results confirm that FIN-14 encodes a late-inducible gene. Moreover, nuclear run-on assays document that FIN-14 is transcriptionally activated in NIH 3T3 cells following growth factor stimulation. Tissue distribution studies indicate expression of a single mRNA species in the kidney with low levels of expression observed in several other tissues including testis and thymus. Mouse embryogenesis studies indicate that FIN-14 expression occurs constitutively in mouse embryos between day 10.5 and 15.5. Unlike NIH 3T3, FIN-14 was constitutively expressed in PC12 cells and its level did not vary appreciably in response to growth factor stimulation. The role of PEGen 21/FIN-14 in progression in E11/E11-NMT model system is not currently known.

30 PEGen 26 is expressed at ~3- to 4-fold higher levels in E11-NMT than in E11 cells. This cDNA is identical to rat poly(ADP-ribose) polymerase (PARP) (39). PARP contributes to the ability of eukaryotic cells to contend with both environmental and endogenous genotoxic agents (40). PARP is a nuclear enzyme that binds to DNA breaks and then catalyzes the covalent modification of acceptor proteins with poly(ADP-ribose) (39,40). PARP activity contributes to the recovery of proliferating cells from DNA damage

and to the maintenance of genomic stability, which may be regulated by effects on chromatin structure, DNA base-excision repair and cell cycle regulation (39,40). The role of PEGen 26/PARP in mediating the progression phenotype is not currently known. However, since cancer is a progressive disease characterized by the accumulation of genetic alterations in the evolving tumor (6), it is tempting to speculate that overexpression of PEGen 26/PARP in E11-NMT may facilitate the ability of these aggressive cancer cells to maintain genomic stability during cancer progression. In this context, PEGen 26/PARP may be an integral component of progression. This hypothesis is readily testable. PEGen 30 is expressed at 2- to 3-fold higher levels in E11-NMT than in E11 cells. This cDNA displays ~98.5% homology to rat *espl* (41). Rat *espl* encodes a 24-kDa nuclear protein which is the rat homologue of *Drosophila* Enhancer of split., a gene involved in ventral ectodermal development in *Drosophila* (41). PEGen 30 appears to be a homologue of *espl*, since the message detected in E11 and E11-NMT cells (~4 kb) is larger in size than the reported *espl* transcript (1.3 kb) (41). The role of PEGen 30/*espl* in tumor progression in E11/E11-NMT model system remains to be determined.

The PSGen cDNAs, 12, 13, 26, 27, 28 and 29, consist of sequences without homology to those in various DNA data bases. Expression of PSGen 12 and PSGen 13 cDNAs is ~3- to 4-fold higher in E11 versus E11-NMT cells (Fig. 29). It is not currently known whether these genes simply correlate with or functionally regulate the progression phenotype. The identification of full-length cDNAs for PSGen-12 and PSGen-13, as well as the other novel PSGen and PEGen cDNAs, are in progress and once isolated experiments can be conducted to directly define the role of these progression-related genes in cancer progression.

Presently demonstrated is a modified gene-identification and gene-cloning technique, RSDD, that can efficiently identify differentially expressed cDNAs. As predicted, subtractive hybridization prior to differential RNA display greatly reduces band complexity, a problem encountered in standard DDRT-PCR in which RNA samples are directly analyzed without subtraction. Unlike a previous report using subtracted cDNAs processed through successive rounds of PCR (25,42), common bands were eliminated using reciprocally subtracted cDNA libraries that had not been processed using PCR. In addition to subtraction hybridization, the discovery of differentially expressed genes was further streamlined by using reverse Northern analyses with isolated cDNAs. With 3 single anchored oligo dT primers and 18 arbitrary 5' primers, 72 bands were identified that displayed differential expression using reverse Northern analysis. Currently, 38 cDNA species have been analyzed by Northern blotting and 31 (~82%) displayed differential expression in E11 versus E11-NMT cells. Sequence analysis of the cloned cDNA fragments revealed 16 different genes, including 11 novel genes not reported in recent DNA databases. RSDD represents a method of choice either as a more efficient and less time consuming modification of the differential RNA display strategy or as a screening methodology for identifying differentially expressed genes in reciprocally subtracted cDNA libraries. Moreover, the ability of RSDD to identify differentially expressed genes that are dissimilar to those recognized using standard DDRT-PCR or subtraction hybridization indicates that this approach will be a valuable adjunct in cloning the complete repertoire of differentially expressed gene changes occurring between complex genomes.

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